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14. ABSTRACT The purpose of this study is to determine the functional role of the putative oncogene BORIS in ovarian cancer. The proposed studies will use a newly developed mouse model system to assess the oncogenic potential of BORIS expression in the ovarian surface epithelium, alone and in combination with Rb and p53 knockout. We have accomplished a number of objectives: i) we have obtained IACUC, ACURO, and Biosafety approvals for the proposed studies, ii) we designed and constructed a new conditional overexpression construct (iZEG-CTCFL) to drive BORIS expression in mice, iii) we obtained double conditional knockout mice (Rb, p53) in FVB/N background and bred with FVB/N wild-type mice, obtained F1 mice and intercrossed to generate stocks of single Rb ^{fl/fl} or p53 ^{fl/fl} mice. iv) we obtained founder BORIS-Tg mice and crossed into the FVB/N strain to fully characterize the transgenic gene configuration, v) we conducted intrabursal Ad-Cre injections to generate cohorts of conditionally deleted Rb and p53 in mice, measured ovary/tumor volume over 300 days by MRI, and confirmed that double mutant mice have significantly enhanced ovary/tumor growth, and vi) Ms. March has learned how to isolate and culture OSE cells from mice and has begun to isolate and utilize these cultures to meet the study objectives.				
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INTRODUCTION

BORIS (CTCFL) is the antagonistic paralog of CTCF, a protein involved in global regulation of chromatin structure and genomic imprinting. In human epithelial ovarian cancer (EOC), BORIS is aberrantly expressed due to promoter DNA hypomethylation. We have previously shown that an increased expression ratio of *BORIS/CTCF* in EOC is associated with global DNA hypomethylation, advanced stage, and reduced survival (Woloszynska-Read et al., *Clinical Cancer Research*, 2011). These findings suggest BORIS activation may be oncogenic in EOC. Based on the known function of CTCF in chromatin insulation, BORIS activation may also remodel the epigenome, particularly via DNA methylation changes (both hypermethylation and hypomethylation). It is plausible that BORIS activation may act cooperatively with Rb and p53 loss to drive epigenetic changes and EOC progression, as BORIS activation in EOC is coincident with activation of E2F target genes, and wildtype p53 is a negative regulator of BORIS expression. To test these hypotheses, we will develop and utilize a murine transgenic model that allows for the specific expression of BORIS in the ovarian surface epithelium (OSE), following delivery of adenovirus expressing Cre recombinase into the ovarian bursa.

BODY

Task 1. The goal of the first task is to determine the impact of BORIS expression, alone and in combination with loss of p53 and/or Rb, on EOC development.

1a. IACUC, ACURO, and Biosafety Approvals

The existing IACUC protocol was amended to permit the studies described in the remainder of the proposal and approved for an additional three years in October of 2012. Moreover, ACURO approval for USAMRMC Proposal Number OC110111, Award Number W81XWH-12-1-0456, entitled "Functional Assessment of the Role of BORIS in Ovarian Cancer Using a Novel in Vivo Model System" was obtained on 01/31/14. Finally, the Institutional Biosafety Protocol for the use of adenoviruses was also resubmitted and approved by Roswell Park Cancer Institute.

1b. Generation of a transgenic mouse model with conditional CTCFL (BORIS) transgene in an FVB/N background

As outlined in the SOW, we decided to generate a new CTCFL (BORIS) transgenic mouse so that it would be in the FVB/N background. In addition, we decided to remake the transgene in the iZEG conditional expression construct instead of the originally used pCLEG vector (described in original proposal) because it contains reporters for both pre-activation (LacZ) and post-activation (EGFP) states. To help prevent epigenetic gene silencing that can take place with randomly inserted transgenes, we attempted to use a newly-developed transcription activator-like effector nucleases (TALENs) designed to target the ROSA26 locus. This approach was unsuccessful so we returned to the more traditional strategy (i.e. pronuclear injection with random integration). However, to reduce the chances of epigenetic silencing, we used a BORIS cDNA with a modified sequence (i.e. no CpG dinucleotides) that cannot be methylated.

Of 25 pups born following pronuclear injection, 4 were found to carry the BORIS transgene. These mice were mated to FVB/N mice and their progeny genotyped by PCR. Two litters showed evidence of germline transmission after genotyping by PCR; however, only one male mouse was confirmed by Southern analysis. Following its mating to several FVB/N females it was determined that the multiple copies of the BORIS cDNA are likely present at a single genomic locus. Characterization of the BORIS-Tg model included the establishment of mouse embryonic fibroblast (MEF) cell lines to confirm the function of the transgene. Following infection of these cells with an adenovirus expressing Cre-recombinase, expression of BORIS was detected, indicating that the transgene functioned as designed and that ectopic expression of BORIS was not toxic at the cellular level. Considering the proposed role of BORIS in reprogramming the

epigenome, inappropriate expression of the BORIS transgene would be expected to result in embryonic lethality or developmental defects. To test this hypothesis, female mice carrying both the BORIS transgene and the Zp3-Cre transgene (expresses Cre-recombinase just before fertilization, resulting in excision of the floxed STOP cassette in oocytes), were mated to FVB/N males. Of more than 80 offspring from these crosses, not a single newborn pup contained the BORIS transgene suggesting that the transgene is expressing a functional BORIS protein and indicating that, as anticipated, ectopic expression of BORIS at fertilization was incompatible with proper development.

1c. Generation of control and experimental mice and intrabursal injection of adenovirus expressing Cre-recombinase

The initial double conditional knockout mouse ($pRb^{flox/flox};p53^{flox/flox}$ in FVB/N background) was bred with FVB/N wild type mice, and F1 mice intercrossed to generate stocks of single $p53^{flox/flox}$ knock out mice. As controls, cohorts of 15 double mutant ($pRb^{flox/flox};p53^{flox/flox}$) and 15 single mutant ($p53^{flox/flox}$) female mice were established by conventional breeding and were treated with adeno-Cre-IRES-eGFP by intrabursal injection. To control for surgery and/or response to viral infection, contralateral ovarian bursa were injected with adenovirus that expresses only eGFP

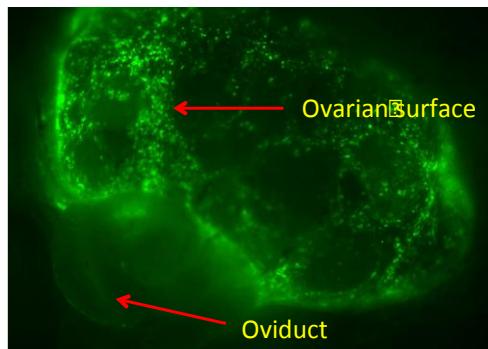


Figure 1. Green fluorescent protein in mouse ovarian surface epithelium (OSE) following intrabursal injection of adenovirus expressing Cre and GFP.

(adeno-eGFP). Now that we have the BORIS transgenic mouse, cohorts including the transgene (e.g. $pRb^{flox/flox};p53^{flox/flox}$; $Tg^{iZEG-CTCFL/+}$) ("triple mutants") are being produced.

Following surgery and intrabursal injection of several test animals, immunofluorescence analysis showed that infection of the OSE was successful (Fig. 1) Control cohorts (i.e. $pRb^{flox/flox};p53^{flox/flox}$

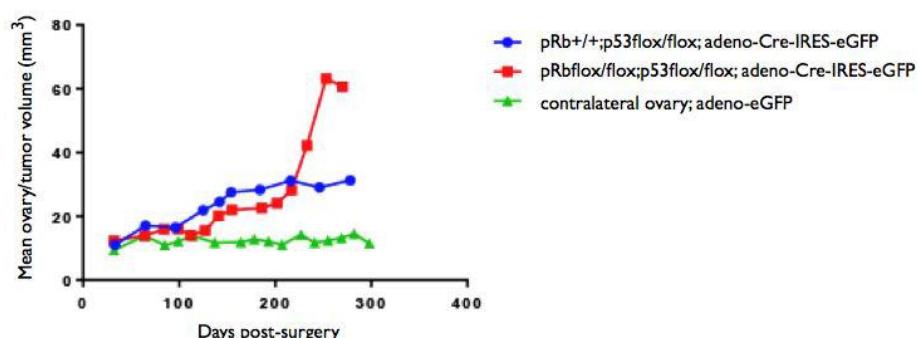


Figure 2. Tumor development in control cohorts. Single mutant ($pRb^{+/+};p53^{flox/flox}$) or double mutant ($pRb^{flox/flox};p53^{flox/flox}$) female mice were subjected to intrabursal injection of adenoviruses expressing Cre-recombinase and eGFP or just eGFP (contralateral ovary), and monitored by MRI for 280-300 days. The graph shows the mean ($n=15$) ovary/tumor volume for the indicated cohort.

and $p53^{flox/flox}$) injected with AdCre alone were monitored by MRI for enlargement of ovaries and/or tumor development. As anticipated, ovary enlargement/tumor development increased dramatically in the double mutant cohort compared to the single mutant cohort (Fig. 2).

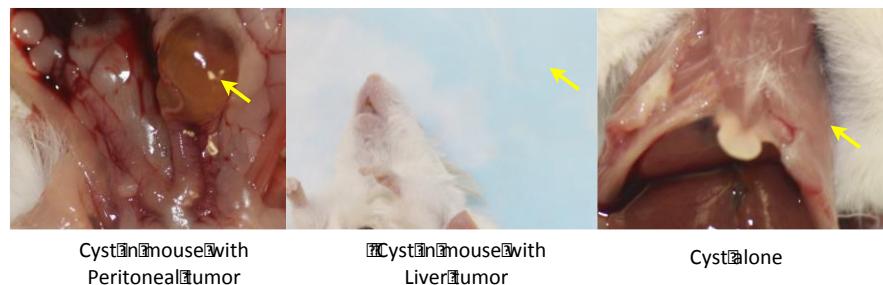


Figure 3. BORIS expression appears to increase the frequency of ovary-associated inclusion cysts (arrow) irrespective of tumor formation or tumor location, reminiscent of the phenotype observed when the TGFB signaling pathway is disrupted (Quartuccio et al. PLOS ONE 8: e65

Alternative strategy to ectopically express BORIS in OSE: Since the generation of the BORIS-Tg mouse line had taken so long, we employed an alternative approach to ectopically express BORIS in the mouse ovarian surface epithelium (OSE). With help from the Iowa University Vector Core Facility, we constructed an adenovirus that expresses human BORIS (Ad-BORIS). Upon infection of several cell types with this virus, robust expression of hBORIS was observed. AdBORIS was co-injected with AdCre-IRES-eGFP into the ovarian bursa of a second cohort of mice (wild type FVB/N, [$pRb^{+/+}$; $p53^{flox/flox}$] and double mutant [$pRb^{flox/flox}$; $p53^{flox/flox}$]) circumventing the need to use the BORIS-Tg mouse line. Monitoring by MRI for more than 1 year revealed significant fluid accumulation inside the ovarian bursa, which was confirmed upon dissection (Figure 3). Fluid buildup was greater in ovaries injected with both Ad-Cre and Ad-BORIS than in contralateral ovaries injected with only Ad-GFP, suggesting an immune response triggered by more than just the adenovirus itself. The increased inflammation may be due to enhanced transformation resulting from ectopic BORIS expression. More importantly, although the experiment is still ongoing, there appears to be a reduction in latency in tumor development and morbidity in mice treated with Ad-Cre + Ad-BORIS compared to those treated with only Ad-Cre (Figure 4), although the reduction in morbidity is just short of statistical significance ($p = 0.058$). Finally, preliminary analysis by pathologists at IDEXX BioResearch suggests that mice treated with Ad-BORIS developed a more diverse spectrum of tumors (i.e. mixture of ovarian and peritoneal with varying epithelial and mesenchymal phenotype).

Task 2. The goal of the second task is to determine the impact of BORIS expression, alone and in combination with loss of Rb and/or p53, on epigenomic and genomic stability in EOC

Similar cohorts of “control” animals (i.e. $pRb^{flox/flox}$; $p53^{flox/flox}$ and $p53^{flox/flox}$) containing 15 female mice each have been established. These mice have all undergone ovarian intrabursal injections of AdCre and sacrificed at various times and tumor material collected for genomic (CGH) and epigenomic (DNA methylation) analysis. Similar cohorts of animals carrying the BORIS-Tg with

or without floxed p53 and/or floxed pRb will be established by *in vitro* fertilization, injected with adeno-Cre, and used for tumor collection.

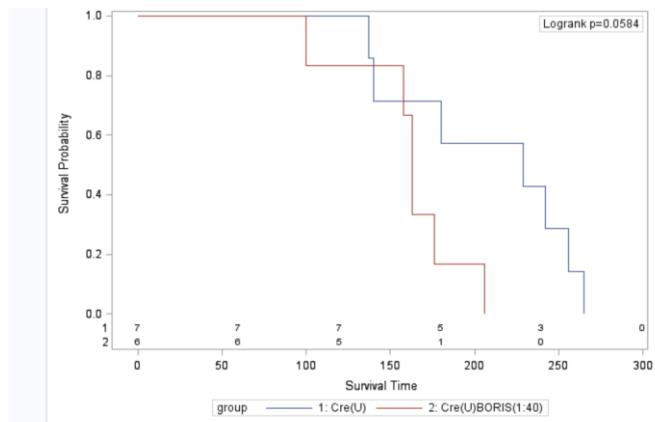


Figure 4. Ectopic BORIS expression reduces the time to morbidity in mouse model of ovarian cancer. Intrabursal injection of AdCre-eGFP alone (5×10^{10} pfu/mL)(blue) or AdCre-eGFP + AdBORIS (1:40) (1.75×10^9 pfu/mL)(red) was performed on 8-12 week old $pRb^{flox/flox};p53^{flox/flox}$ female mice in estrus. Estrus was determined by vaginal cytology. The group injected with both viruses succumbed to tumors earlier than those injected with AdCre-eGFP alone. Median time to death, control: 229 days post-surgery. Median time of death, experimental: 163 days post-surgery.

Task 3. The goal of the third task is to determine the impact of BORIS expression, alone and in combination with loss of p53 and/or Rb, on OSE transformation in vitro.

While visiting Dr. Flesken-Nikitin at Cornell, **Ms. Joanna Hillman March (Teal Scholar)** learned to isolate and culture ovarian surface epithelial (OSE) cells. She has successfully established OSE cultures from double conditional knockout mice ($pRb^{flox/flox};p53^{flox/flox}$) as well as from $p53^{flox/flox}$ knock-out mice. As described above, we have now generated the BORIS-Tg mice as originally proposed in the SOW. These mice are currently being bred to mice of various genotypes ($pRB^{fl/fl}$, $p53^{fl/fl}$, $pRB^{fl/fl};p53^{fl/fl}$), to determine the oncogenic properties of OSE cells harvested from mice with all genetic configurations. These experiments will include testing for growth in soft agar and invasive growth through Matrigel.

Emerging evidence suggests that at least some ovarian tumors arise from fallopian tube epithelial cells (Reade et al. *J Obstet Gynaecol Can* 36:133, 2014). We have therefore obtained the FT282 cell line from Dr. Ronny Drapkin, University of Pennsylvania, which are immortalized, but non-tumorigenic, cells derived from fallopian tube epithelium (Karst et al. *Mol Cellular Pathology* 74:1141, 2013). Since anecdotal evidence suggests that excessive ectopic expression of BORIS can lead to protein inclusion bodies and cell death, **Ms. Joanna Hillman March (Teal Scholar)** has generated an cimate-inducible BORIS lentivirus system to test whether titrated levels of ectopic BORIS expression can transform these cells as assessed by both cell culture experiments (e.g. soft agar and invasive growth through Matrigel), and by tumorigenicity experiments (xenografts in SCID mice).

KEY RESEARCH ACCOMPLISHMENTS

- Obtained IACUC, ACURO, and Biosafety approvals for all proposed studies.

- Designed and constructed a new conditional overexpression construct (iZEG-CTCFL) to drive BORIS expression in mice.
- Obtained double conditional knockout mice (Rb, p53) in FVB/N background and bred with FVB/N wild-type mice, obtained F1 mice and intercrossed to generate stocks of single Rb-flox or p53-flox mice.
- Obtained founder BORIS-Tg mice and found that multiple copies of the transgene have integrated into a single genomic locus. This novel transgenic mouse strain has been partially characterized by showing (1) that the transgene is inducible following Cre-recombination mediated excision of the floxed STOP cassette, (2) ectopic expression of human BORIS in MEFs is not toxic at the cellular level but (3) induced expression during early development causes embryonic lethality, possibly by disrupting normal epigenetic reprogramming.
- Obtained “triple mutant” mice in FVB/N background that carry floxed p53 and Rb genes plus the inducible BORIS transgene.
- Conducted intrabursal Ad-Cre injections to generate cohorts of conditionally deleted Rb and p53 in mice, measured ovary/tumor volume over 300 days by MRI, and confirmed that double mutant mice have significantly enhanced ovary/tumor growth.
- Conducted intrabursal Ad-BORIS plus Ad-Cre injections to generate cohorts of conditionally deleted Rb and p53 in mice that express BORIS in the OSE, although this experiment has not reached endpoint, it appears that ectopic expression of BORIS results in increased (perhaps transformation-related) inflammation, a reduction in tumor latency, and a more diverse tumor phenotype.
- **Ms. Joanna Hillman March (Teal Scholar)** has learned how to isolate and culture OSE cells from mice and has begun to isolate and utilize these cultures to meet the study objectives. She has also generated an inducible lentivirus expression system in FT282 immortalized human fallopian tube epithelial cells to test the effect of different levels of BORIS expression on transformation.

REPORTABLE OUTCOMES

A. Publications, Abstracts, and Presentations

Publications:

Barger, C., Zhang, W., Hillman, J., Stablewski, A. B., Higgins, M., Vanderhyden, B. C., Odunsi, K., and A. R. Karpf. (2015). Genetic determinants of FOXM1 overexpression in epithelial ovarian cancer and functional contribution to cell cycle progression. *Oncotarget*, 6(29), 27613-27.

Joanna Hillman, Aimee Stablewski, Nicholas Sass, Adam R. Karpf *, Michael Higgins (2016). Ectopic expression of BORIS alters tumor phenotype in ovarian cancer mouse model. Manuscript in Preparation.

Abstracts:

Barger, C., Zhang, W., Odunsi, K., and A. R. Karpf. 2015. FOXM1 expression in epithelial ovarian cancer coincides with gene amplification and functional loss of p53 and Rb, and drives G2-M progression and target gene expression. Midwest Student Biomedical Research Forum. February 28. Omaha, NE.

Barger, C., Zhang, W., Odunsi, K., and A. R. Karpf. 2015. FOXM1 expression in epithelial ovarian cancer coincides with gene amplification and functional loss of p53 and Rb, and drives G2-M progression and target gene expression. AACR Annual Meeting. April 18-22. Philadelphia, PA.

Hillman, J., Karpf, A. R., and M. J. Higgins. (2016). Ectopic expression of BORIS alters tumor phenotype in ovarian cancer mouse model. Roswell Park Scientific Retreat July 21-22, 2016.

Lectures:

Adam Karpf: *FOXM1/RHNO1: a new oncogenic axis in epithelial ovarian cancer*. Nebraska Center for Cell Signaling/Department of Oral Biology. University of Nebraska at Lincoln. Lincoln, NE. December 2, 2015.

Adam Karpf: *FOXM1/RHNO1: a new oncogenic axis in epithelial ovarian cancer*. Ovarian Cancer Research Center. University of Pennsylvania. Philadelphia, PA. February 24, 2016.

B. Degrees obtained supported by this award:

Progress towards the completion of the Ph.D. degree for: Mr. Carter Barger, University of Nebraska Medical Center, Cancer Biology Research Program. Anticipated graduation date: May 2017.

Progress towards the completion of the Ph.D. degree for: **Ms. Joanna Hillman March (Teal Scholar)**, University at Buffalo Roswell Park Cancer Institute, Cancer Genetics Graduate Program. Anticipated graduation date: September 2016.

C. Funding received

UNMC Assistantship/Fellowship Award, 2015-2017. *Forkhead Box M1 (FOXM1) in High Grade Serous Ovarian Cancer (HGSOC)*. Student: Carter Barger.

D. Funding Applied For

NIH/NCI RO1

07/01/16 – 06/31/21

FOXM1/RHNO1 in High Grade Serous Ovarian Cancer

Role: PI (Karpf)

Direct Costs:

The goal of this project is to characterize a novel oncogenic axis in ovarian cancer and to assess whether it is a therapeutic vulnerability in this disease.

DOD Ovarian Cancer Research Program

05/01/16 – 04/31/19

FOXM1/RHNO1: A Novel Oncogenic Module in High Grade Serous Ovarian Cancer

Role: PI (Karpf)

Direct Costs:

The goal of this project is to characterize a novel oncogenic module in ovarian cancer and to study whether it presents a novel means to achieve synthetic lethality in effected tumors.

RPCI-UPCI Ovarian Cancer SPORE Developmental Research Program

Can BORIS drive fallopian tube-derived ovarian cancer?

07/01/16 – 06/30/17

Role: PI (Higgins)

Direct Costs:

The goal of this project is to determine whether ectopic expression of BORIS in the mouse oviduct (fallopian tube) can cooperate with deficiencies in p53 and/or Rb to drive ovarian tumorigenesis.

CONCLUSION

We have made significant progress towards functionally assessing the role of BORIS in epithelial ovarian cancer. We have completed the necessary experimental design, have trained with the necessary experimental methods, and are currently producing mice to test the central hypotheses of the proposal. Successful completion of this proposal will reveal important new information

about the molecular pathology of ovarian cancer, and the role of aberrant BORIS expression in the disease. This knowledge can be used to develop novel biomarkers and therapeutic approaches for ovarian cancer.

APPENDICES

1) Publication: Barger, C., Zhang, W., Hillman, J., Stablewski, A. B., Higgins, M., Vanderhyden, B. C., Odunsi, K., and A. R. Karpf. (2015). Genetic determinants of FOXM1 overexpression in epithelial ovarian cancer and functional contribution to cell cycle progression. *Oncotarget*, 6(29), 27613-27.

2) Abstract: Joanna Hillman, Aimee Stablewski, Nicholas Sass, Adam R. Karpf *, Michael Higgins (2016). Ectopic expression of BORIS alters tumor phenotype in ovarian cancer mouse model. Manuscript in Preparation.

SUPPORTING DATA

N/A

Genetic determinants of FOXM1 overexpression in epithelial ovarian cancer and functional contribution to cell cycle progression

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ABSTRACT

The FOXM1 transcription factor network is frequently activated in high-grade serous ovarian cancer (HGSOC), the most common and lethal subtype of epithelial ovarian cancer (EOC). We used primary human EOC tissues, HGSOC cell lines, mouse and human ovarian surface epithelial (OSE) cells, and a murine transgenic ovarian cancer model to investigate genetic determinants of FOXM1 overexpression in EOC, and to begin to define its functional contribution to disease pathology. The Cancer Genome Atlas (TCGA) data indicated that the *FOXM1* locus is amplified in ~12% of HGSOC, greater than any other tumor type examined, and that *FOXM1* amplification correlates with increased expression and poor survival. In an independent set of primary EOC tissues, *FOXM1* expression correlated with advanced stage and grade. Of the three known *FOXM1* isoforms, *FOXM1c* showed highest expression in EOC. In murine OSE cells, combined knockout of *Rb1* and *Trp53* synergistically induced FOXM1. Consistently, human OSE cells immortalized with SV40 Large T antigen (IOSE-SV) had significantly higher FOXM1 expression than OSE immortalized with hTERT (IOSE-T). FOXM1 was overexpressed in murine ovarian tumors driven by combined *Rb1*/*Trp53* disruption. FOXM1 induction in IOSE-SV cells was partially dependent on E2F1, and FOXM1 expression correlated with E2F1 expression in human EOC tissues. Finally, FOXM1 functionally contributed to cell cycle progression and relevant target gene expression in human OSE and HGSOC cell models. In summary, gene amplification, p53 and Rb disruption, and E2F1 activation drive FOXM1 expression in EOC, and FOXM1 promotes cell cycle progression in EOC cell models.

INTRODUCTION

Approximately 70% of EOC cases are diagnosed at advanced stage; long-term survival for these patients is poor and has not improved significantly in the past three decades [1, 2]. Current clinical management of EOC is surgical debulking and adjuvant chemotherapy using a platinum-taxane doublet. While the majority EOC patients are initially responsive to chemotherapy, most patients relapse and current second line therapies

are not curative. Increased knowledge of the pathological and genetic underpinnings of EOC and HGSOC, its most common and lethal subtype, are likely to lead to advances in diagnosis and treatment [3]. For example, TCGA recently reported mRNA and miRNA expression, DNA copy number alterations (CNA), DNA promoter methylation, and mutational data for HGSOC, which led to classification into sub-groups based on these molecular criteria [4]. CNA is prominent in HGSOC, and occurs at a higher frequency than in any other TCGA-profiled tumor

type [4–6]. It was also notable that *TP53* was mutated in virtually all HGSOC, suggesting p53 as a “gatekeeper” for this disease [4]. Other tumor suppressors and oncogenes implicated in HGSOC include BRCA1/2, Rb, PI3K, Ras, and CCNE1 [4, 7–9]. Finally, *FOXM1* pathway activation is a highly frequent alteration in HGSOC, second only to *TP53* mutation [4].

FOXM1 is a member of the Forkhead box (FOX) transcription factor family, which is unified by a conserved winged helix DNA binding motif [10]. The binding specificity of *FOXM1* relative to other family members is in part achieved via an atypical chromatin interaction mechanism in which *FOXM1* is bridged to DNA by the Myb-MuvB (MMB) transcriptional activator complex [11]. At least two important biological pathways are influenced by the transcriptional activity of *FOXM1*: cell cycle (G1-S and G2-M transitions), and DNA damage (homologous recombination DNA repair) [10, 12]. *FOXM1* is overexpressed and activated in many human cancers and possesses oncogenic activity *in vitro* and *in vivo* [12]. Mechanisms accounting for *FOXM1* overexpression in cancer cells and tissues are diverse and include p53, Rb, and *FOXO3* loss [13–16], Myc, HIF-1, Gli1, SP1, STAT3 and E2F activation [17–22], and gene amplification [23].

Human *FOXM1* has 10 exons with alternative splicing of exons Va (A1) and VIIa (A2), giving rise to three *FOXM1* variants: *FOXM1a*, *FOXM1c*, and *FOXM1b*. *FOXM1a* contains exons Va and VIIa, with the latter disrupting the transactivation domain, making this isoform transcriptionally inactive [24]. *FOXM1* expression was reported to be restricted to dividing cells with onset of expression at late G1 and peak expression at G2-M [25, 26]. *FOXM1* protein is additionally regulated throughout the cell cycle via phosphorylation [10]. Once activated, *FOXM1* can promote cell cycle progression through transactivation of target genes, leading to progression through both G1-S [27–29] and G2-M [30–32] checkpoints.

The goal of the current study was to begin to define the genetic determinants of *FOXM1* overexpression in EOC, to analyze its expression during disease progression, and to investigate its role in EOC cell cycle progression. For this task, we utilized publically available EOC databases, primary human EOC tissues, immortalized ovarian surface epithelial (OSE) cell models (murine and human), a transgenic murine ovarian cancer model, and human HGSOC cell lines. Together, our data implicate gene amplification, Rb and p53 inactivation, and E2F1 activation in *FOXM1* overexpression in EOC. Among *FOXM1* isoforms, *FOXM1c* showed highest expression in EOC cells and tumors. *FOXM1* was overexpressed in late-stage, high-grade disease, and *FOXM1* gene amplification correlated with reduced HGSOC survival. Finally, we demonstrated that *FOXM1* contributes to cell cycle progression in OSE and HGSOC cell models.

RESULTS

***FOXM1* gene amplification correlates with increased *FOXM1* expression and reduced survival in HGSOC**

FOXM1 is located at chromosome 12p13.33, a known amplified region in cancer [23, 33, 34]. We thus examined *FOXM1* copy number in TCGA datasets using cBioPortal [35, 36]. Notably, amongst all tumor types with TCGA data, *FOXM1* was most frequently amplified in HGSOC, with ~12% of tumors effected (Figure 1A). Together, over half of HGSOC cases showed either copy number gains or amplifications, suggesting *FOXM1* as an HGSOC oncogene (Figure 1B). To determine if *FOXM1* copy number status correlates with expression, we compared *FOXM1* mRNA expression and copy number in TCGA HGSOC data. We observed a progressive increase in *FOXM1* expression with copy number status that was highly significant (Figure 1C). We additionally compared overall survival (OS) to *FOXM1* CNA and *FOXM1* expression, and observed that the former showed a significant correlation with OS, while the latter did not (Figure 1D and data not shown). This finding suggests that additional genes located at the amplified region of 12p13.33 may contribute to OS in HGSOC, and/or that *FOXM1* protein or activation levels may be more relevant than mRNA levels for impacting OS. Finally, our analysis of TCGA mutational data did not reveal *FOXM1* mutations in HGSOC (data not shown).

***FOXM1* expression in relation to EOC type and progression status, and *FOXM1* isoform expression in EOC**

We next examined *FOXM1* expression using an independent set of EOC tissues with diverse histology, stage, and grade [37, 38]. RT-qPCR analysis demonstrated that *FOXM1* is frequently overexpressed in different EOC histological subtypes relative to normal ovary (NO), and furthermore shows increased expression in both late-stage and high-grade disease (Figure 2A–2C). While we did not have mRNA from normal fallopian tube available for analysis, it is notable that the TCGA reported low expression of *FOXM1* mRNA in normal fallopian tube as compared to HGSOC (see FigS10.4 in [4]). Similar to the mRNA, *FOXM1* protein expression was elevated in EOC as compared to NO (Figure 2D). *FOXM1* has three known splice variants: *FOXM1a*, *b*, and *c*, which encode proteins with varying activities [24]. We used isoform specific RT-qPCR and found that *FOXM1c* is the predominant isoform expressed in EOC, followed by *FOXM1b* and *FOXM1a* (Figure 2E).

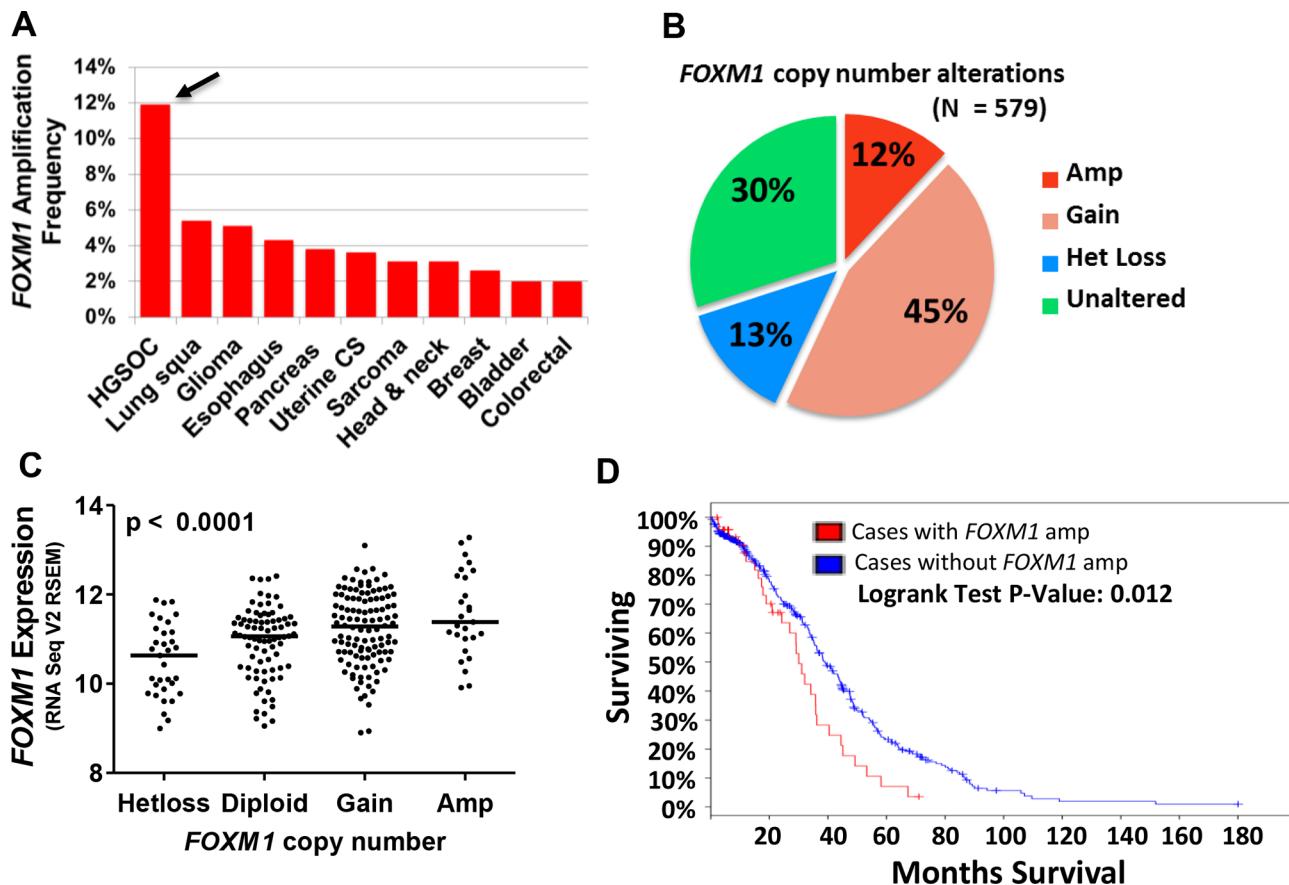


Figure 1: FOXM1 copy number alterations (CNA) in HGSOC. **A.** FOXM1 amplification frequency in TCGA datasets. Arrow indicates HGSOC. **B.** FOXM1 CNA in HGSOC TCGA datasets as determined by GISTIC. **C.** FOXM1 expression (RNA Seq V2 RSEM, log2) compared to FOXM1 copy number in HGSOC TCGA datasets. The *p* value for ANOVA with post-test for linear trend is shown. Lines represent group medians. **D.** Overall survival as a function of FOXM1 amplification in HGSOC TCGA datasets. The *p* value for Logrank test is shown.

FOXM1 expression in HGSOC cell models

We used clinically relevant cell models of human HGSOC to examine genetic influences on FOXM1 expression [39]. All cell lines used have *TP53* mutations as well as additional genetic alterations relevant to HGSOC (Figure 3A). We found that *FOXM1* mRNA expression was elevated in all but one cancer cell line as compared to hOSE cells, and was heterogeneous in the HGSOC cell types (Figure 3B). Notably, highest *FOXM1* expression was observed in the two cell lines (SNU-119, COV362) in which the *FOXM1* locus is amplified. Isoform-specific RT-qPCR revealed highest expression of *FOXM1c*, moderate expression of *FOXM1b*, and lowest expression of *FOXM1a* in HGSOC cell lines. *FOXM1c* expression was highest in the SNU-119 and COV362 lines, in which *FOXM1* is amplified (Figure 3C). The relative expression of the three *FOXM1* isoforms is in agreement with our primary tumor data (Figure 2E).

Disruption of Rb and p53 induces FOXM1 expression in murine and human OSE cells

The OSE is a potential tissue of origin for EOC, and primary OSE cells are useful for exploring EOC relevant processes [40, 41]. We first used established murine OSE (mOSE) cell models to examine mechanisms regulating FOXM1 expression. We focused on *TP53* and *Rb1*, as mutations or disruptions in these genes are frequent in HGSOC [4, 9]. *Trp53* and *Rb1* knockout was achieved through Ad-Cre infection of mOSE cells as described previously (Figure 4A) [42]. While loss of either tumor suppressor gene (TSG) alone resulted in a modest upregulation of *Foxm1*, combined p53 and Rb loss led to robust induction (Figure 4B). Similar effects were observed for FOXM1 protein expression (Figure 4C). We next investigated the potential role of p53 and Rb in FOXM1 regulation in human OSE (hOSE) cells by measuring FOXM1 expression in hOSE cells immortalized

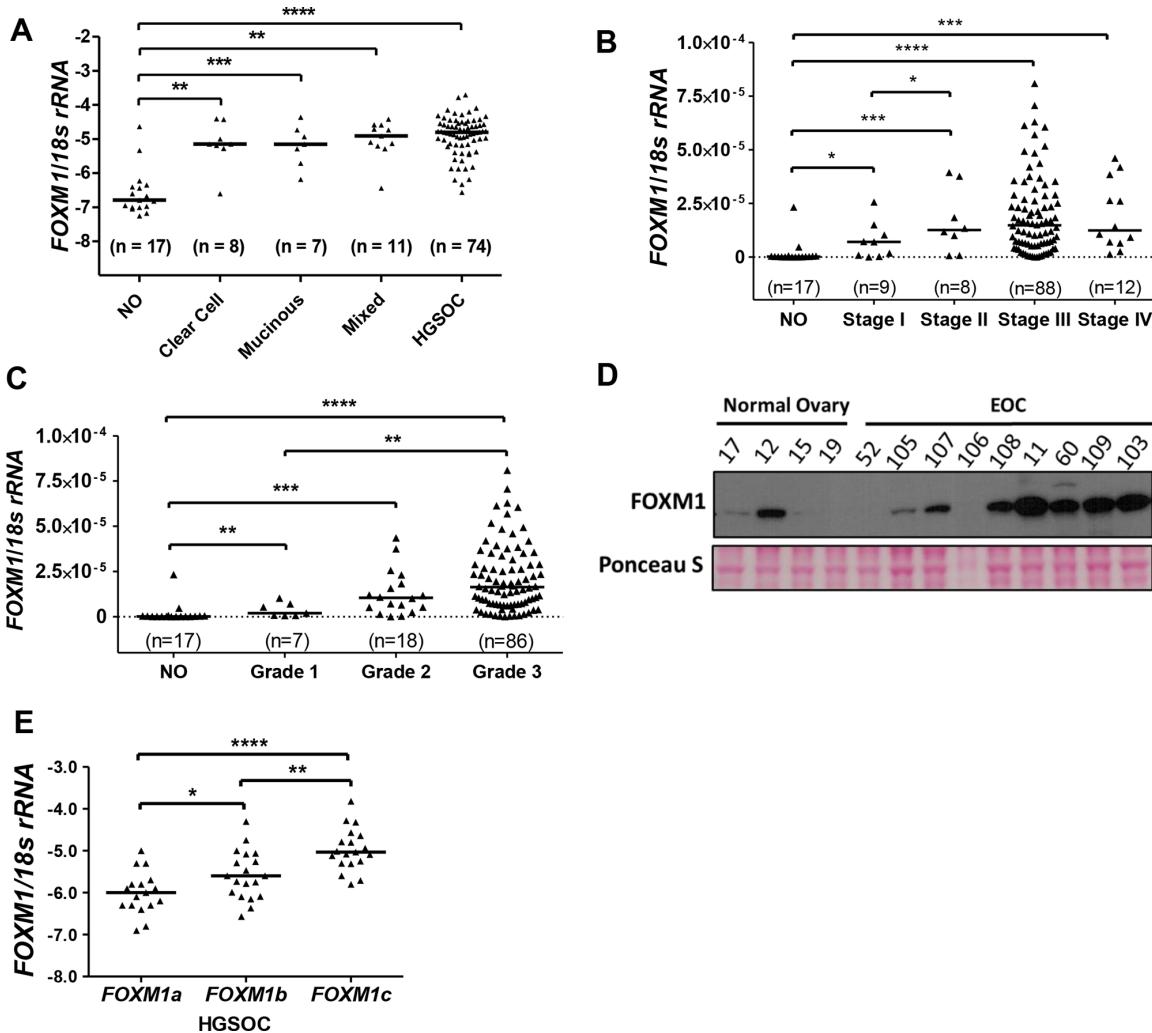


Figure 2: FOXM1 expression in EOC. **A.** *FOXM1* expression measured with RT-qPCR (log10) in EOC histological subtypes as compared to normal ovary (NO). *FOXM1* expression was normalized to *18s rRNA*. **B.** *FOXM1* expression in NO and in EOC as a function of disease stage. **C.** *FOXM1* expression in NO and in EOC as a function of pathological grade. Lines represent group medians. Mann-Whitney test *p* values are shown. **D.** *FOXM1* Western blot analysis in NO and EOC. Ponceau S staining is shown as a loading control. **E.** *FOXM1* isoform specific RT-qPCR (log10) measured in HGSOC tissues. Lines represent group medians. The Mann-Whitney test *p* value is shown. *p* value designation: **** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05.

with either SV40 Large T antigen (IOSE-SV), which leads to potent inactivation of p53 and Rb, or hTERT (IOSE-T), which leaves both proteins intact [43]. IOSE-SV cells showed significantly higher levels of expression of both *FOXM1* mRNA and protein as compared to IOSE-T or primary (non-immortalized) human OSE cells (Figure 4D-4E). These data suggest that Rb and p53 play a major role in regulating *FOXM1* expression in OSE cells.

FOXM1 is overexpressed in murine ovarian cancer driven by combined p53/Rb1 disruption

To complement the OSE cell studies, we measured *FOXM1* expression in murine ovarian tumors developing after dual disruption of p53 and Rb in the OSE (see *Methods*). As shown in Figure 5A-5B, *FOXM1* mRNA and

protein expression were significantly increased in ovarian tumors as compared to the mouse normal ovary control. These *in vivo* data provide further support that loss of p53 and Rb contribute to *FOXM1* overexpression in ovarian cancer. Notably, immunohistochemistry (IHC) analyses of the ovarian tumors arising in this model indicated that the tumors were negative for cytokeratin expression and positive for smooth muscle actin (Figure 5C). This finding suggests that cancer in this model may represent leiomyosarcoma, and not EOC, as reported previously [42].

E2F1 and FOXM1 expression in OSE cells and EOC

Transcriptional activation of *FOXM1* following Rb loss suggests that E2F transcription factors may contribute

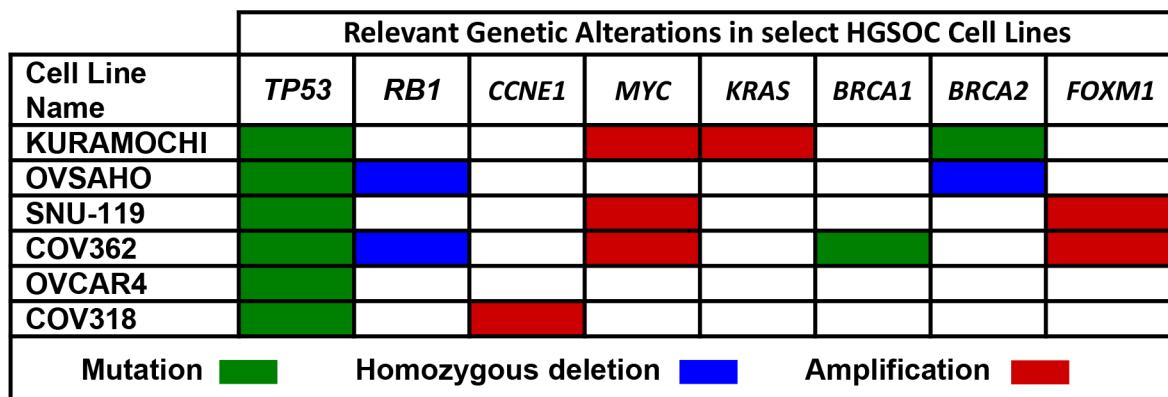
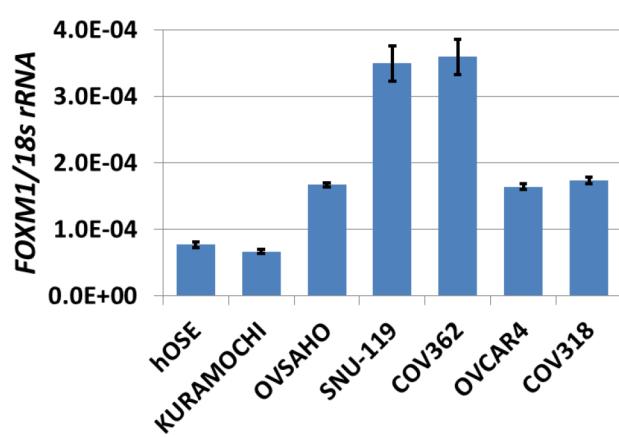
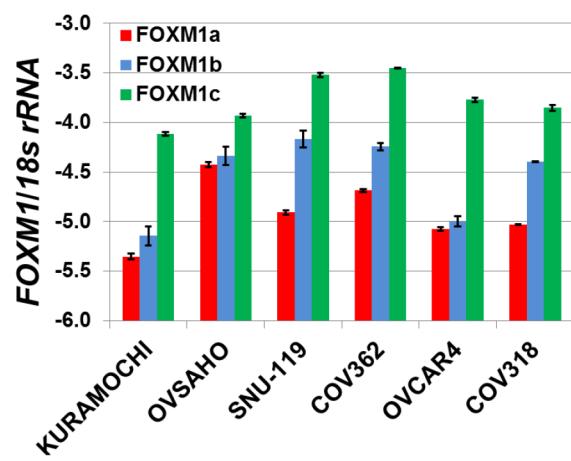
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Figure 3: FOXM1 expression in HGSOC cell lines. **A.** Relevant genetic alterations in HGSOC cell lines. Data were retrieved from CCLE and copy number alterations were visualized with IGV as described in *Methods*. **B.** Pan-*FOXM1* mRNA expression in HGSOC cell lines and hOSE cells (control) was measured by RT-qPCR. **C.** Isoform specific *FOXM1* mRNA expression in HGSOC cell lines was measured by RT-qPCR (log10). For B–C, bars represent mean \pm SD.

to FOXM1 overexpression. To test this, we used IOSE-SV and COV362 cells, which have high FOXM1 expression as well as alterations in p53 and Rb. Following E2F1 knockdown by siRNA (Figure 6A), *FOXM1* mRNA expression in both cell types was significantly reduced, as compared to the non-targeting siRNA control (Figure 6B). To validate this finding in the primary disease setting, we tested whether *FOXM1* correlates with *E2F1* expression in human EOC. As shown in Figure 6C–6D, in both the TCGA HGSOC dataset and in our independent set of EOC tissues, expression of *FOXM1* and *E2F1* were highly correlated. Together, these data implicate E2F1 in promoting FOXM1 expression in EOC.

Functional contribution of FOXM1 to EOC cell cycle progression and target gene expression

To determine if FOXM1 plays a functional role in EOC cells, we explored its canonical function in cell cycle progression using knockdown and overexpression

approaches. Knockdown of FOXM1 was efficient in IOSE-SV cells (Figure 7A), and led to accumulation of cells in G2-M, with concomitant decreases in both G1 and S (Figure 7B; representative histograms shown in Supplementary Figure 1A). In COV362 cells, FOXM1 knockdown also led to decreased cells in S phase, but caused accumulation of cells in G1 with no significant alteration of G2-M (Figure 7D–7E; representative histograms shown in Supplementary Figure 1B). To determine whether the observed effect of FOXM1 knockdown on cell cycle progression coincided with altered expression of relevant FOXM1 target genes, we analyzed *SKP2*, *PLK1*, and *CCNB1* expression. *SKP2* promotes G1-S transition, while *PLK1* and *CCNB1* promote G2-M transition, both downstream of FOXM1 [28, 31, 44]. In agreement with our cell cycle data, FOXM1 knockdown in IOSE-SV and COV362 downregulated these genes, with the lone exception of *PLK1* in COV362 (Figure 7C, 7F).

In addition to FOXM1 knockdown, we overexpressed FOXM1b or FOXM1c using a stable doxycycline (Dox)-inducible system in primary hOSE cells. Interestingly,

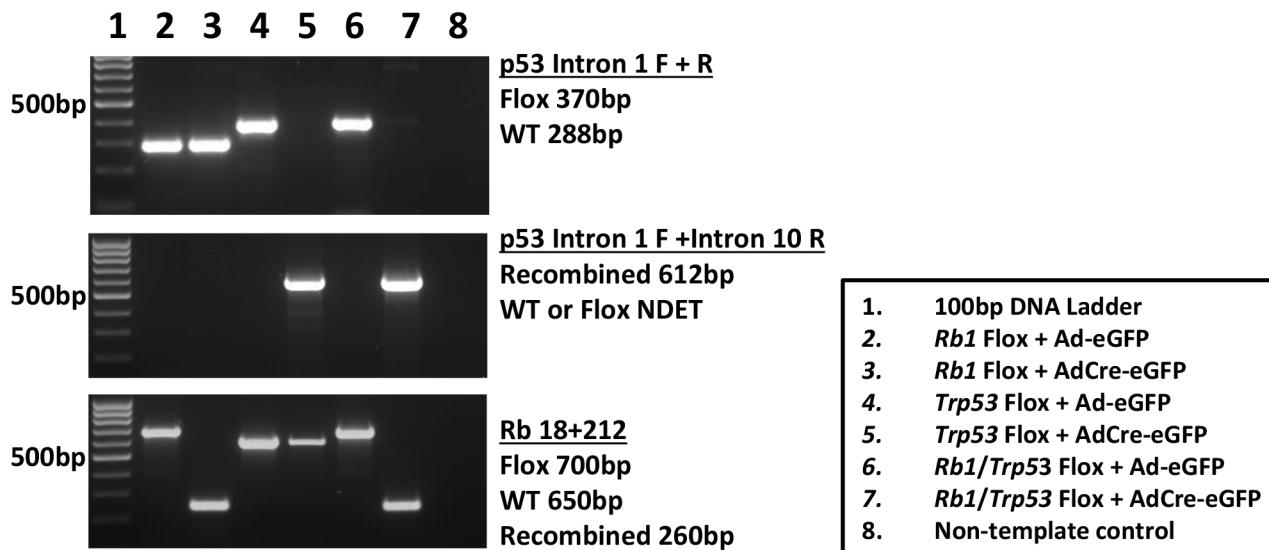
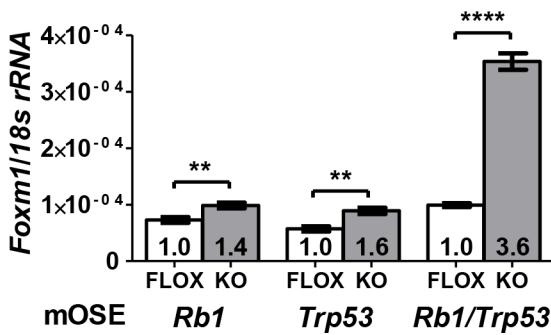
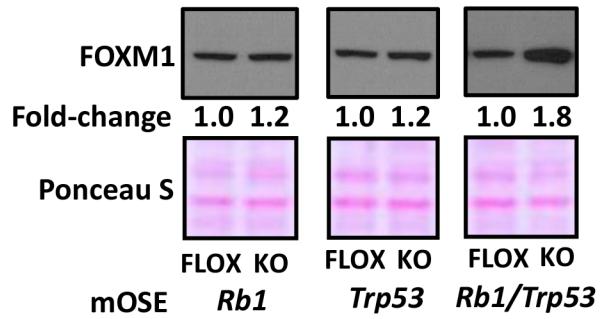
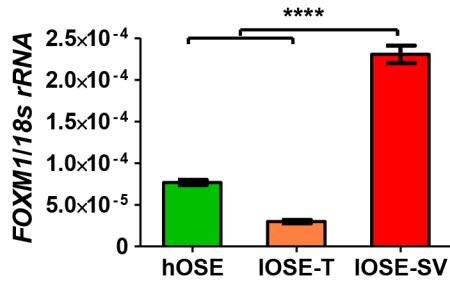
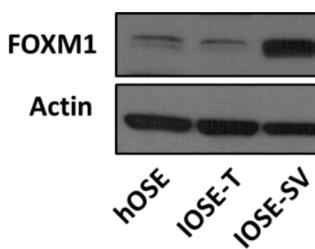
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Figure 4: FOXM1 expression in murine and human OSE cells following Rb and/or p53 abrogation. **A.** PCR genotyping of mOSE cells following infection with recombinant adenovirus expressing enhanced GFP (Ad-eGFP, control) or Cre recombinase + eGFP (AdCre-eGFP). **B–C.** FOXM1 expression in Rb and/or p53 floxed (control) and knockout (post-Cre infection) mOSE cells. **B.** *Foxm1* RT-qPCR with respective fold-change relative to the floxed control. Data represents mean \pm SD. Students *t*-test *p* value is shown. **C.** FOXM1 Western blot with respective fold change relative to the floxed control, performed with nuclear lysates. Ponceau S staining is shown as a loading control. **D–E.** FOXM1 expression in primary and immortalized human OSE cells (hOSE, IOSE-T, IOSE-SV). Cell line descriptions are provided in the *Methods*. **D.** *FOXm1* RT-qPCR. Data represent mean \pm SD. **E.** FOXM1 Western blot. β -actin is shown as a loading control. Students *t*-test *p* values: **** < 0.0001 , *** < 0.001 , ** < 0.01 , * < 0.05 .

while the mRNA expressions were identical, the FOXM1c protein appeared to be more stable than FOXM1b in these cells (Figure 8A–8B). FOXM1c overexpression in hOSE led to increased cells in S and G2/M, with a decrease in G1

(Figure 8C). In contrast, overexpression of FOXM1b did not alter cell cycle (data not shown). To determine whether the effect of FOXM1c overexpression on cell cycle coincided with altered expression of relevant FOXM1 target genes, we

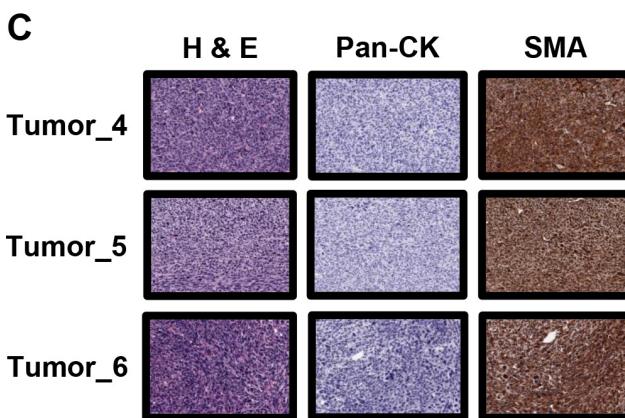
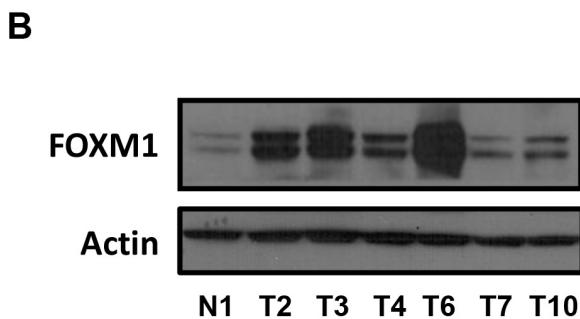
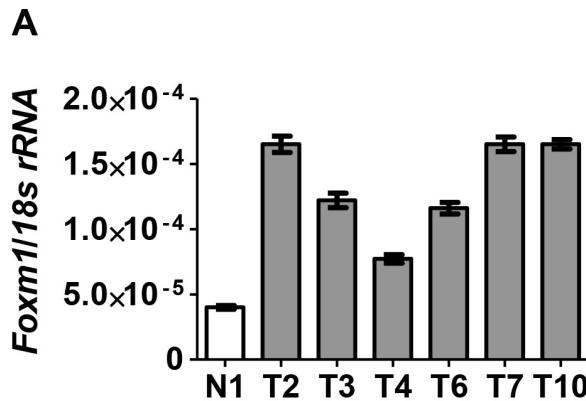


Figure 5: FOXM1 expression in Rb1/Trp53 knockout-driven murine ovarian cancer. A–B. FOXM1 expression in *Rb1/Trp53* knockout murine ovarian tumor tissues (T) and murine normal ovary control tissue (N). The mouse model is described in *Methods*. A. *Foxm1* RT-qPCR. Data represents means \pm SD. B. FOXM1 Western blot. β -actin is shown as a loading control. C. Ovarian tumor histology in *Rb1/Trp53* knockout mice. Paraffin sections of the tumors were stained with H&E or specific antibodies to pan-cytokeratin (Pan-CK) or smooth muscle actin (SMA). Images were captured using 20X magnification. Antigen detection is indicated by the presence of a brownish-red stain.

again analyzed *SKP2*, *PLK1*, and *CCNB1*. Overexpression of FOXM1c in hOSE cells led to upregulation of *PLK1* and *CCNB1*, while *SKP2* was unaffected (Figure 8D). These data are consistent with the functional impact of FOXM1 in EOC cell cycle regulation, and suggest that this activity may be mediated by FOXM1's function as a transcriptional regulator. Although the effect of FOXM1c overexpression on cell cycle was modest, this could be due to the primary hOSE cell model used. In agreement, the effect of FOXM1 overexpression on cell cycle progression in primary hOSE are reminiscent of that reported in cancer cells, although the effects were more robust in the latter [45, 46].

DISCUSSION

Several mechanisms have been reported to contribute to FOXM1 overexpression in cancer, including gene amplification, loss of negative regulation by p53, Rb, and FOXO3, and transcriptional activation by E2F and Myc [13–17, 22, 23]. To date, the mechanisms underlying FOXM1 upregulation in HGSOC have not been described, although FOXM1 pathway activation is extremely frequent in this malignancy. Here we demonstrate that, in EOC, *FOXM1* is upregulated at the transcriptional level by combined loss of Rb and p53, and show that FOXM1 copy

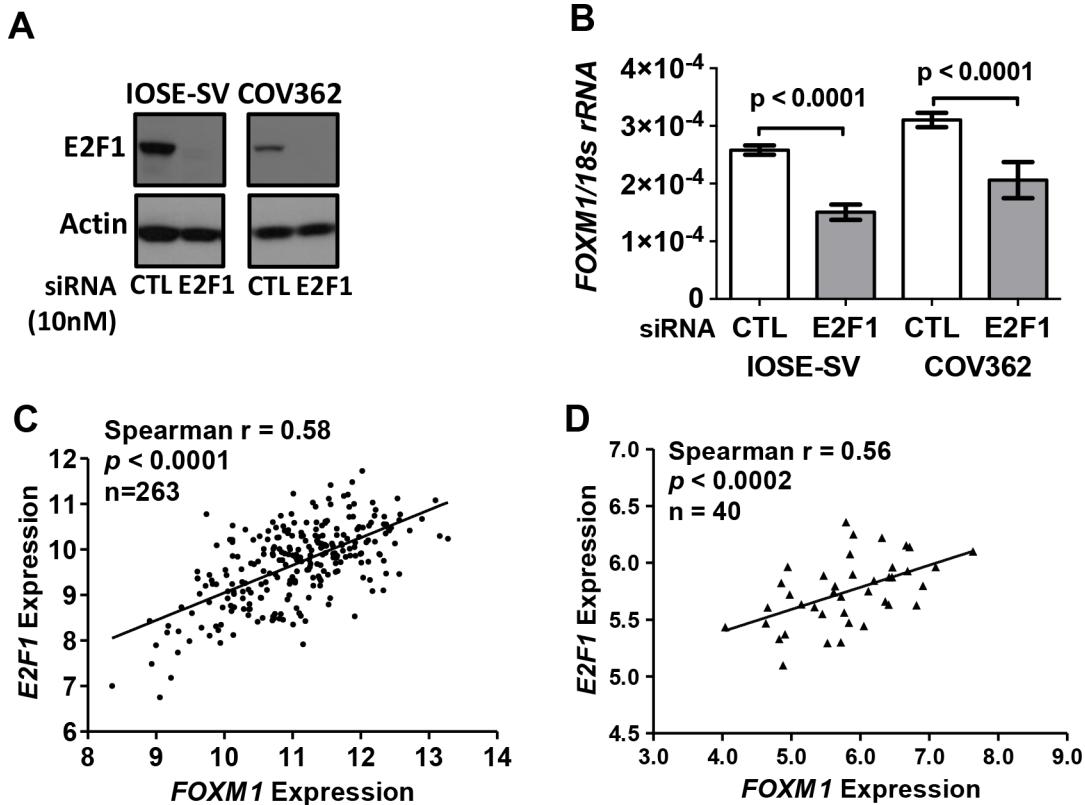


Figure 6: E2F1 and FOXM1 expression in IOSE-SV cells, HGSOC cells, and primary tumors. A–B. siRNA knockdown of E2F1 (10 nM) in IOSE-SV and COV362 cells for 72 hours. A. E2F1 Western blot. β -actin is shown as a loading control. B. *FOXM1* RT-qPCR, normalized to *18s rRNA*. Data represent mean \pm SD. Student's *t*-test *p* value is shown. C–D. *E2F1* and *FOXM1* expression correlation in human EOC. C. Correlation in 263 HGSOC tissues from TCGA datasets (gene expression determined by RNA seq V2, log2). D. Correlation in an independent set of 40 EOC tissues (gene expression determined by Affymetrix HG 1.0ST microarray, log2).

number gains correlate with increased FOXM1 expression in primary tumors and cell lines. Combinatorial loss of p53 and Rb in murine and human OSE cells synergistically induced FOXM1 expression, and murine ovarian cancer arising in a p53/Rb compound deletion model led to FOXM1 overexpression. In addition, we demonstrate that E2F1 contributes to FOXM1 overexpression in cell models, and closely correlates with FOXM1 expression in primary tumors. Thus, our data establish p53 and Rb as negative regulators, and E2F1 and copy number gain as positive regulators, of FOXM1 expression in EOC. Consistent with our p53 data, it was recently shown that Nutlin 3-mediated p53 activation repressed FOXM1 in EOC cells [47].

We observed that p53 and Rb loss cooperatively drive high level FOXM1 expression in EOC relevant cell models, and our data reveal E2F1 as a factor contributing to this induction. Several potential mechanisms may underline these observations. First, loss of Rb function leads to activation of E2F transcription factors [48], and two putative E2F sites have been identified in the FOXM1 promoter [22]. Second, the Rb-E2F pathway is regulated by p21, a potent negative regulator of cyclin-dependent kinases (CDK) and a direct transcriptional target of p53.

Therefore, functional loss of p53 may relieve p21-mediated repression of E2F1, which in turn may promote FOXM1 expression. In agreement, prior work shows that p53-mediated repression of FOXM1 is partially p21-dependent [14, 22]. p53-mediated negative regulation of FOXM1 may also be independent of effects on the Rb-E2F pathway, although this remains to be determined. In addition to p53, Rb, and E2F1, other relevant mechanisms of FOXM1 induction involve Myc and FOXO3 [16, 17]. These may act independently or in concert with p53 and Rb loss, and require further study using EOC models.

Importantly, a recent study observed increased FOXM1 staining in early precursor Serous Tubal Intraepithelial Carcinoma (STIC) lesions, and showed that FOXM1 expression was maintained in invasive tumors [16]. As *TP53* mutations appear to be a ubiquitous early event in human HGSOC, we speculate that during HGSOC tumor progression, loss of Rb function and/or *FOXM1* amplification, coupled with the p53 impairment already present, leads to high level FOXM1 expression. Consistent with this model, our data indicate that FOXM1 expression is markedly elevated in late stage, high-grade EOC. Further verification of this model requires determination of FOXM1 protein expression during EOC disease progression.

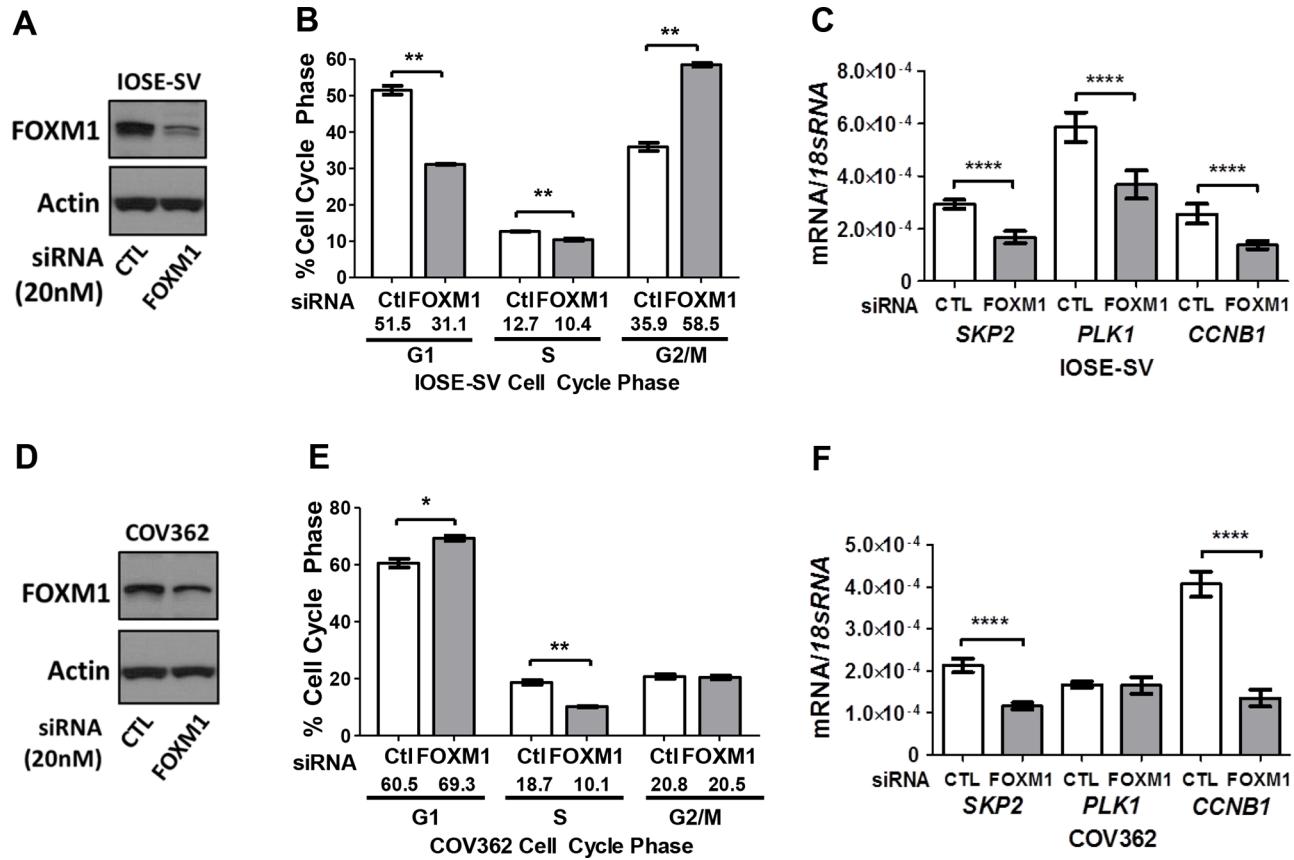


Figure 7: Impact of FOXM1 knockdown on cell cycle progression and target gene expression in IOSE-SV and COV362 cells. Transient siRNA-mediated knockdown of FOXM1 (20 nM) was completed for 72 hours. **A.** Validation of FOXM1 protein knockdown in IOSE-SV cells. FOXM1 protein expression was determined by Western blot, and β -actin is shown as a loading control. **B.** Cell cycle analysis of IOSE-SV cells following FOXM1 or control siRNA treatment. **C.** FOXM1 target gene expression determined by RT-qPCR in IOSE-SV cells, following FOXM1 or control siRNA treatment. Expression data are shown for *SKP2*, *PLK1*, and *CCNB1*, each normalized to *18s rRNA*. **D-F.** Same as A-C, except the experiment was performed using COV362 cells. Bars represent mean \pm SD. Student's *t* test *p* values are shown. *P* value designation: **** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05.

We found that the predominant *FOXM1* isoform expressed in HGSOC is *FOXM1c*. An earlier study showed that *FOXM1c* is the predominant isoform expressed in pancreatic cancer, while another study showed that *FOXM1b* is the major isoform expressed in other cancer types [20, 49]. *FOXM1c* has alternative exon A1; residues in this region can be phosphorylated by the RAF/MEK/MAPK signaling cascade, providing a distinction with *FOXM1b* [50]. Considering the differential expression and functional potential of different *FOXM1* isoforms, it is important to determine which variants are responsible for the oncogenic activity in EOC. In this context, our cell cycle data suggests that *FOXM1c*, but not *FOXM1b*, drives cell cycle progression in hOSE cells. Notably, a recent study discovered the expression of additional isoforms of *FOXM1* in ovarian cancer, and speculated that these isoforms may be constitutively active [51].

In the current study, we demonstrated a role for *FOXM1* in cell cycle progression using primary and immortalized human OSE cells and using a HGSOC cell line. Beyond its role in cell cycle progression,

FOXM1 has been shown to contribute to other important oncogenic phenotypes in ovarian cancer, including platinum and taxane resistance, epithelial-to-mesenchymal transition (EMT), cell migration, and cell invasion [52–55]. Additionally, it is plausible that *FOXM1* overexpression, combined with p53 gain of function mutations, may synergistically promote genomic instability in EOC. For example, *FOXM1* upregulation induced genomic instability in normal human keratinocytes, and *FOXM1* is a member of a conserved gene expression profile for genomic instability in human cancer [56, 57]. Furthermore, p53 gain of function mutations can positively regulate *FOXM1* and correlate with higher levels of genomic instability as compared to p53 null mutations [47, 58]. Based on the functions of *FOXM1* that have been described, it is likely that *FOXM1* contributes to multiple oncogenic phenotypes during HGSOC genesis and progression, including genomic instability in early STIC lesions, EMT in primary tumors, and metastatic tumor growth and drug resistance in late stage disease.

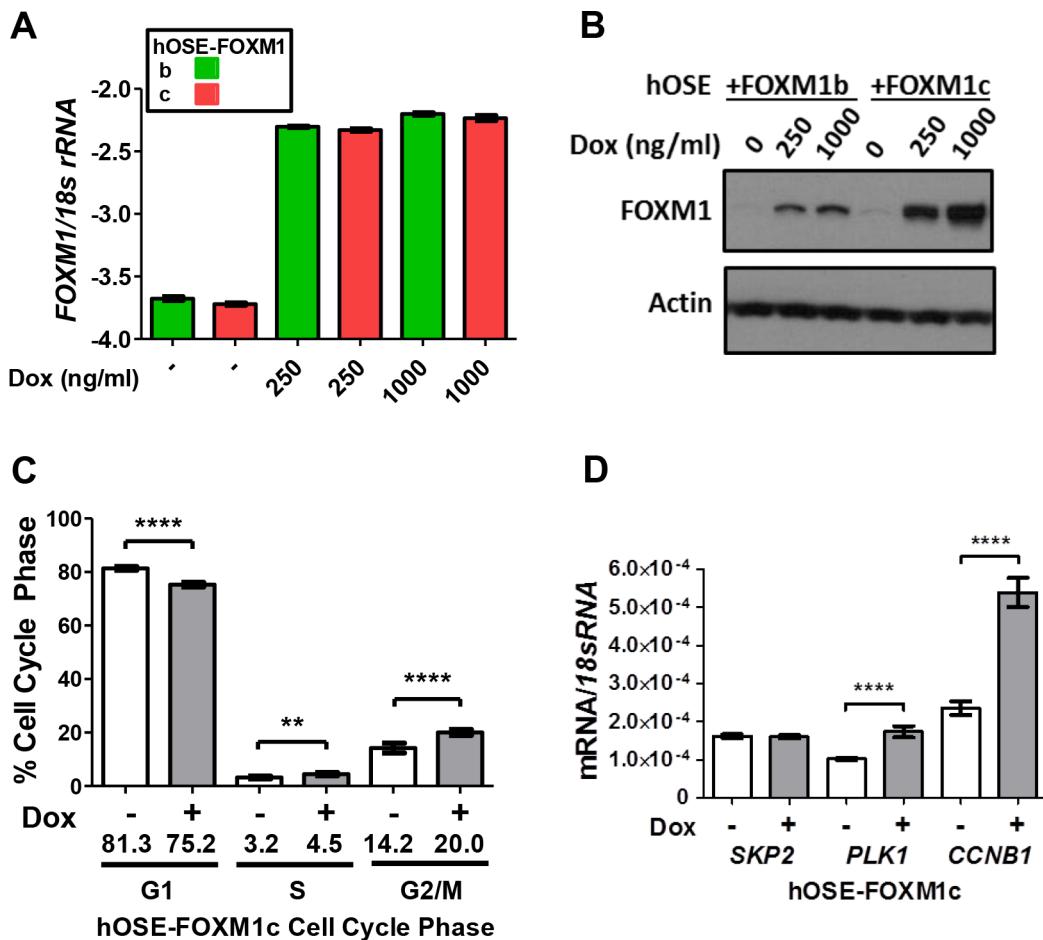


Figure 8: Impact of FOXM1 overexpression on cell cycle progression and target gene expression in hOSE cells. **A–B.** Dox-inducible FOXM1b and FOXM1c overexpression in primary hOSE cells after 72 hours of doxycycline treatment as indicated. **A.** *FOXM1* RT-qPCR (log10). **B.** FOXM1 Western blot. β -actin is shown as a loading control. **C.** Cell cycle analysis following Dox-inducible FOXM1c overexpression in primary hOSE cells after 72 hours of treatment. Cells treated with 250 ng/ml and 1000 ng/ml doxycycline were combined for analysis and compared against the control without treatment. **D.** FOXM1 target gene expression was measured by RT-qPCR in hOSE cells following 72 hours of doxycycline treatment to induce FOXM1c. Expression data are shown for *SKP2*, *PLK1*, and *CCNB1*, each normalized to *18s rRNA*. Data represents mean \pm SD. Student's *t*-test *p* values are shown. *P* value designation: **** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05.

Based on the oncogenic role of FOXM1 in cancer, there is significant interest in developing drugs that target this protein. This is particularly relevant in HGSOC, for which current therapeutic regimens, especially for late-stage disease, are inadequate. Until recently, available FOXM1 inhibitors, i.e. the thiazole antibiotics Siomycin A and Thiomestrepton, were non-specific and had global effects on proteasome-dependent pathways [59, 60]. However, a recent paper identified and characterized a specific inhibitor of FOXM1, FDI-6 [61]. FDI-6 was reported to specifically inhibit the DNA binding activity of FOXM1, but not other FOX family members, and was shown to inhibit cancer cell growth *in vitro*. FDI-6 needs additional validation, as concerns have been raised about its specificity [62], and its potency may require improvement for possible treatment of FOXM1-dependent cancers. Despite these caveats, the existence of a small

molecule inhibitor of FOXM1 provides a new and exciting opportunity to pursue relevant translational studies in FOXM1-dependent cancers, including HGSOC.

MATERIALS AND METHODS

The cancer genome atlas (TCGA) data analysis

TCGA provisional data was retrieved from BioPortal on January 5, 2015. All provisional cancer datasets were analyzed for FOXM1 mutation and somatic copy-number alterations. The genomic profile of FOXM1 was further analyzed in the HGSOC (Ovarian Serous Cystadenocarcinoma-TCGA Provisional) dataset for putative somatic copy-number alterations from GISTIC [63], using Onco Query Language (OQL), and mRNA expression (RNA seq V2 RSEM). GISTIC predicts gene

copy number alterations according to sample specific thresholds generated by comparing chromosomal segments with median chromosomal arm copy numbers. High gains (Amp) are segments with copy number that exceed the maximum median chromosomal arm copy number for that sample by at least 0.1; low gains (Gain) are segments with copy numbers from 2.1 to the high gain threshold; neutral segments (Diploid) have copy numbers between 1.9 and 2.1; shallow losses (Hetloss) have copy numbers between 1.9 and the deep deletion threshold; and deep deletions (Homdel) have copy numbers that are below the minimum median chromosomal arm copy number for that sample by at least 0.1. Overall patient survival was determined by Kaplan-Meier Survival. E2F1 mRNA expression (RNA seq V2 RSEM) was retrieved from the same dataset. All parameters were set at default.

Human primary tissues

Normal ovary (NO) and epithelial ovarian cancer (EOC) tissues were obtained from patients undergoing surgical resection at Roswell Park Cancer Institute (RPCI) under Institutional Review Board-approved protocols, and were described previously [37, 38]. Frozen tissues were processed for biochemical extractions as described previously [37, 38].

Reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA was purified using TRIzol (Invitrogen) and quality was determined by RNA denaturing gel. Briefly, one μ g of RNA was DNase-treated using the DNA-free kit (Ambion), and converted to cDNA using the iScript cDNA synthesis kit (BioRad). One μ l of 1:5 cDNA sample dilutions were used for qPCR reactions. Standard curves were prepared using gel-purified end-point RT-PCR products. All samples were run in triplicate, and all gene expression data were normalized to *18s rRNA*. PCR was performed with an annealing temperature of 60°C and a total of 45 cycles for all primer pairs. Dissociation curves were performed to confirm specific product amplification. RT-qPCR standards for each gene were generated from a mixture of human or mouse cell cDNA via end point RT-PCR. Gradient PCR reactions were used to optimize annealing temperatures for each primer set. Primer sequences are listed in Supplementary Table S1.

Western blot analyses

Whole cell protein extracts were prepared with RIPA buffer [1X PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] supplemented with protease and phosphatase inhibitors (Sigma), and centrifuged at 4°C for 10 minutes at 14000g. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific)

supplemented with protease and phosphatase inhibitors. Protein concentration was determined by the BCA protein assay (Thermo Scientific). Equal amounts of protein (30–50 μ g) were fractionated on 4–12% gradient SDS-polyacrylamide gel electrophoresis gels (Invitrogen) and transferred to PVDF membrane (Roche). Membranes were stained with Ponceau S to confirm efficient transfer and equal loading then blocked with 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) for 1 hour at room temperature. Membranes were incubated with primary antibodies in 5% nonfat dry milk in TBST at 4°C overnight followed by incubation with secondary antibody in 5% nonfat dry milk in TBST for 1 hour at room temperature. The following primary antibodies, purchased from Santa Cruz Biotechnology, were used at the indicated dilutions: FOXM1 (sc-500; 1:500, sc-271746; 1:500), E2F1 (sc-251; 1:500), β -Actin (sc-47778; 1:5000). Enhanced chemiluminescence (Thermo Fisher Scientific) was used for protein detection. Quantification of protein expression was performed using ImageJ software (Image Processing and Analysis in Java, National Institute of Health) [64].

The Cancer Cell Line Encyclopedia (CCLE)

The copy-number and mutational profiles of CCLE cell lines KURAMOCHI, SNU-119, OVSAHO, COV362, COV318 and OVCAR4 were visualized using the Integrative Genomics Viewer (IGV, version 1.4.2.) [65].

Cell culture

COV362 and COV318 cell lines (Sigma) were cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Life Technologies), 1% penicillin-streptomycin (pen-strep, Life Technologies). KURAMOCHI and OVSAHO (Japanese Collection of Research Bioresources Cell Bank) and SNU-119 (Korean Cell Line Bank) cell lines were cultured in RPMI-1640 (Hyclone) supplemented with 10% FBS and 1% pen-strep. OVCAR4 cells (National Cancer Institute Division of Cancer Treatment and Diagnosis Cell Line Repository) were cultured in RPMI-1640 supplemented with 10% FBS and 1% pen-strep. Primary hOSE cells (ScienCell) were cultured in Ovarian Epithelial Cell Medium (ScienCell, 7311). IOSE-T (a.k.a. IOSE-21, hOSE immortalized with hTERT) cells [43] were a generous gift from Professor Francis Balkwill (Cancer Research UK) and were cultured in Medium 199/MCDB105 (1:1, Sigma) supplemented with 15% FBS, 1% pen-strep, 10 ng/mL human epidermal growth factor (Life Technologies), 0.5 μ g/mL hydrocortisone (Sigma), 5 μ g/mL bovine insulin (Cell Applications), 34 μ g protein/mL bovine pituitary extract (Life Technologies). IOSE-SV (a.k.a. IOSE-121, hOSE immortalized with SV40 Large T antigen) cells were a generous gift from Dr. Nelly Auersperg (University of British Columbia) and were cultured in Medium 199/MCDB105 (1:1)

supplemented with 10% FBS and 25 µg/ml gentamicin (Life Technologies). mOSE cells [42] were a generous gift from Professor Barbara Vanderhyden (Ottawa Hospital Research Institute) and were cultured in Alpha Modified MEM (Corning) containing 10% FBS, 0.05% pen-strep, 1 µg/ml gentamicin, and 1% insulin–transferrin–sodium–selenite solution (ITSS, Roche). HEK293T cells (American Type Tissue Culture Collection) were cultured in DMEM with 10% FBS and 1% pen-strep. All cell lines were maintained at 37°C in a humidified incubator with 5% CO₂. Cell culture medium was changed every 3–5 days depending on cell density. For routine passage, cells were split at a ratio of 1:3–10 when they reached 85% to 90% confluence.

Adenoviral transduction of mOSE cells and p53/Rb genotyping

Recombinant adenovirus expressing enhanced GFP (Ad-eGFP, control), or both eGFP and Cre recombinase (AdCre-eGFP), were purchased from the University of Iowa Gene Transfer Vector Core. mOSE cells were transduced at an MOI of 200 for 6 hours. Media was changed and cells were allowed to expand for 10 days before harvesting for analysis. Genomic DNA was isolated using the Puregene Tissue Kit (Qiagen). DNA was re-suspended in Tris-EDTA (50 mM, pH 6.8). Genotyping for *p53* and *Rb* genes were performed as previously described [42]. PCR was performed with an annealing temperature of 60°C and 30 cycles for all primer pairs. Primer sequences are listed in Supplementary Table S1.

Murine ovarian cancer transgenic model

Trp53^{loxP/loxP}/*Rb1*^{loxP/loxP} mice (floxed *Trp53* and *Rb1*) were a kind gift from Professor Kenneth Gross (RPCI). All mice were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee (RPCI). Intrabursal injections of recombinant adenovirus expressing both enhanced GFP and Cre recombinase (AdCre-eGFP) or eGFP alone (Ad-eGFP) as the contralateral control (University of Iowa Gene Transfer Vector Core) were performed on adult mice in estrus as previously described [66]. Mice were determined to be in estrus by vaginal cytology. The original viral stock solution was diluted with PBS to 3.5 × 10⁹ pfu/mL immediately before injection of 10 µL. Mice were euthanized and subjected to necropsy when tumor mass exceeded 1 cm or the animal exhibited other signs of sickness, such as abdominal distension and moribund behavior. Tumor samples were dissected and snap frozen in liquid nitrogen and stored at -80°C. Frozen tumor samples were ground into a powder with a mortar and pestle over liquid nitrogen and immediately processed for RNA (miRNeasy Mini Kit, Qiagen) and whole cell protein cell extracts were prepared with RIPA buffer (1X PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors,

and centrifuged at 4°C for 10 minutes at 14000g. RNA was treated for contaminating DNA using the TURBO DNA-free Kit (Ambion), and integrity was determined using a bioanalyzer (Agilent). One µg of DNase-treated RNA was converted to cDNA using the iSCRIPT cDNA Synthesis Kit (Bio-Rad).

Immunohistochemistry

Formalin-fixed paraffin blocks were cut into 4 µm sections, placed on charged slides, and dried at 60°C for one hour. Slides were cooled to room temperature, deparaffinized in three changes of xylene, and rehydrated using graded alcohols. For antigen retrieval, slides were heated in a steamer for 20 min in citrate buffer pH = 6 (BioCare Medical, B910) for Smooth Muscle Actin or target retrieval solution pH = 9 (Dako, S2367) for Cytokeratin and allowed to cool for 20 min, endogenous peroxidase quenched with aqueous 3% H₂O₂ for 10 minutes and washed with PBS/T. Slides were loaded on a Dako autostainer and serum free protein block (Dako, X0909) was applied for 5 minutes, blown off and the corresponding antibody was applied. Smooth Muscle Actin antibody (Abcam, ab5694) was applied at 1:125 (Rabbit IgG) for one hour. Powervision poly HRP anti-rabbit IgG (Leica; catalog #PV6119) was then applied for 30 minutes. L-DAB (Leica; catalog #PV6126) applied for 5 minutes, was used for chromogen visualization. Pan-Cytokeratin antibody (Dako, Z0622) was applied at 1:1750 (Rabbit IgG) for one hour. Rabbit Envision/labeled polymer HRP anti-rabbit (Dako; catalog #K4003) was then applied for 30 minutes. DAB (Dako; catalog #K3468) applied for 10 minutes, was used for chromogen visualization. Lastly, the slides were counterstained with Hematoxylin, rinsed, and cover slipped.

E2F1 and FOXM1 siRNA knockdown

siRNAs (Supplementary Table S1) were transfected with Lipofectamine RNAiMax reagent (Life Technologies), according to the manufacturer's instructions, using the concentrations indicated in the Figures. The non-targeting siRNA #2 (Dharmacon) was used as a control as described previously [67]. 72 hours after transfection, cells were prepared for total RNA and protein extractions, and cell cycle analyses.

Microarray analysis of FOXM1 and E2F1 expression

Affymetrix HG 1.0ST arrays were used to determine the expression of *FOXM1* and *E2F1* in EOC. Probe generation, array hybridization, and expression analyses were performed by the Next Generation Sequencing and Expression Analysis Core Facility at the University at Buffalo Center for Excellence in Bioinformatics. Samples included 40 EOC.

Cell cycle analyses

Cell cycle analysis was performed utilizing the Muse Cell Analyzer (EMD Millipore) and Cell Cycle Assay Kit (EMD Millipore), following the manufacturer's instruction. Briefly, sub-confluent cells were trypsinized, washed with PBS, and filtered with 37 μ m mesh cap tube then fixed in ice-cold 70% ethanol while vortexing and stored 18–24 hours at -20°C. Cells were then stained for 30 minutes at room temperature with propidium iodide (PI) containing RNase, and immediately processed for cell cycle analysis. Representative DNA content histograms are shown in Supplementary Figure S1.

Inducible FOXM1 expression in hOSE cells

The tetracycline-inducible lentiviral pCW57.1-HA-FOXM1b and pCW57.1-DDK-FOXM1c vectors were generated by subcloning human *FOXM1b* and *FOXM1c* from pCMV6 (Origene: SC112825 and SC128214) into pCMV6-AN-HA or -AN-DDK plasmids (Origene: PS100013 and PS100014), respectively, then subcloning into the pCW57.1 (Addgene: 41393) with Gateway cloning (Life Technologies). All plasmids were sequence verified. Replication-deficient lentivirus expressing Dox-inducible FOXM1 was produced by transient transfection of 6.0 μ g psPAX2 (Addgene: 12260), 2.0 μ g pMD2.G (Addgene: 12259), and 8.0 μ g transfer plasmid into HEK293T cells in a 10-cm dish with Lipofectamine 2000 reagent (Life Technologies), according to the manufacturer's instructions. Viral supernatants were collected at 48 hours, passed through a 0.45- μ m filter, and titered by serial dilution with puromycin (Life Technologies) selection and colony formation. The highest dilution producing drug selected colonies was used to transduce primary hOSE cells in the presence of polybrene (4 μ g/ml, Sigma), and 0.5 μ g/ml puromycin was introduced 48 hours post-infection. After five days of puromycin selection, cells were allowed to recover and expand for one week. Cells were seeded in 6-well plates and the next day media was changed with or without doxycycline (Sigma) to induce transgene expression. Media with or without doxycycline was changed every 24 hours. After 72 hours, cells were prepared for total RNA and protein extractions, and cell cycle analyses.

Statistical analyses

Student's *t*-test was used to compare differences between means between two groups. Mann-Whitney test was used to compare differences between medians between two groups. One-way analysis of variance (ANOVA) with a post-test for linear tend was used to compare two or more groups. For all analyses, significance was inferred at $p < 0.05$ and p values were two-sided. Graphpad Prism statistical software (GraphPad Software, Inc).

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CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

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Ectopic expression of BORIS alters tumor phenotype in an ovarian cancer mouse model

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Ovarian cancer is the most lethal gynecological malignancy, claiming the lives of approximately 15,000 American women every year. Ovarian cancer, specifically epithelial ovarian cancer (EOC) is all too often diagnosed at an advanced stage, when tumors become chemoresistant. Understanding the molecular mechanisms involved in the progression of EOC is critical to make any headway in our goal of eradicating this terrible disease. *BORIS* (*CTCFL*) is a cancer-testes antigen (CTA), normally expressed in the testes and aberrantly expressed in various human malignancies, including EOC. We have previously shown a connection between *BORIS* expression and hypomethylation, advanced stage, and poor prognosis in EOC. However, further elucidation of the molecular mechanism employed by *BORIS* is necessary to exploit for cancer treatment. Using a *pRb*^{flox/flox};*p53*^{flox/flox} ovarian tumor mouse model, the impact of *BORIS* expression in conjunction with inactivation of the two tumor suppressor genes was carried out by intrabursal injection of an adenovirus expressing Cre-recombinase (AdCre) alone, or in combination with an adenovirus expressing a human *BORIS* cDNA (AdBORIS). We hypothesized that activation of *BORIS* would result in reduced tumor latency and time to morbidity, and perhaps a greater incidence of metastases. The results suggest that ectopic *BORIS* expression does in fact decrease overall time to death, but did not result in an increased incidence of metastasis when compared to the *p53* and *Rb* inactivated mice alone. Surprisingly, we also observed a shift in the type of tumor when *BORIS* was expressed such that mice injected with AdCre and AdBORIS were more likely to develop peritoneal masses with no overt ovarian tumor than those injected with AdCre alone. We are currently processing fixed tumor samples to determine whether they are of mesenchymal or epithelial origin using H&E staining as well as IHC to known differentiators including smooth muscle actin, cytokeratin, E-cadherin and N-cadherin. Using qPCR gene expression arrays, we are also testing the expression of more than 90 genes associated with the "cancer stem cell" phenotype to determine if tumors generated in mice injected with *BORIS* adenovirus express these genes at a higher level than those from mice not exposed to the *BORIS* adenovirus. These findings could be clinically relevant, as many advanced ovarian carcinomas metastasize to the peritoneal cavity.